AD_____

Award Number: DAMD17-00-1-0395

TITLE: Neoplastic Consequences of a Mutator Phenotype in Human Breast Epithelial Cells: A Prospective Analysis

PRINCIPAL INVESTIGATOR: Kristin A. Eckert, Ph.D.
Danny R. Welch, Ph.D.

CONTRACTING ORGANIZATION: The Pennsylvania State University
College of Medicine
Hershey, Pennsylvania 17033-0850

REPORT DATE: July 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

1230 114

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Annual (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE
Neoplastic Consequences of a Mutator Phenotype in Human Breast Epithelial Cells: A Prospective Analysis

5. FUNDING NUMBERS
DAMD17-00-1-0395

6. AUTHOR(S)
Kristin A. Eckert, Ph.D.
Danny R. Welch, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Pennsylvania State University College of Medicine
Hershey, Pennsylvania 17033-0850

E-Mail: kae4@psu.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Overexpression of DNA polymerase ß in the breast epithelial cell line, MCF-10A, resulted in loss of proliferative potential, most probably due to the upregulation of pro-apoptotic proteins, such as Bax. The *ras*-transformed MCF-10AT cell line is more tolerant of polß overexpression, possibly due to the increased levels of Bcl-2 protein in these cells. However, the MCF-10AT cells continue to display a progressive loss of proliferative potential resulting from polß overexpression. We observed an alteration in MCF-10AT cell phenotype from an adherent to a floating cell morphology after polß overexpression. This is a novel observation and may be related to underlying genomic changes resulting from increased polß protein levels. MCF10AT cells overexpressing polß displayed a tumor incidence and latency similar to parental cells. Thus, in contrast to what we observe in cell culture, a subpopulation of polß overexpressing cells retains proliferative potential *in vivo*. We hypothesize that biologic selection occurs for a mutant, MCF-10AT/WTpolß cell variant within the mouse environment. The requisite genetic variation may be related to the adherent to floating morphology change. The observations that the MCF-10AT/polß tumors are sac-like rather than solid, and that animals bearing MCF-10AT/polß tumors progressed to form lung metastases, further supports our hypothesis.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, genetic instability, mutation, neoplastic evolution, DNA repair, carcinogenesis, tumor progression	10
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

The goal of this proposal is to develop an *ex vivo/in vivo* experimental system that will test whether: (i). a mutator phenotype is *sufficient* to accelerate neoplastic transformation of an immortalized breast epithelial cell line, and (ii). a mutator phenotype is *sufficient* to accelerate malignant progression of a pre-neoplastic cell line. In this approach, overexpression of variant mutator forms of DNA polymerase ß (polß) acts as a surrogate means of generating genetic diversity. Pol ß is the major DNA synthesizing enzyme in base excision repair (BER). DNA lesions resulting from normal cellular metabolism contribute to spontaneous mutations, and are removed by the BER pathway. We hypothesize that intracellular overexpression of polß variant enzymes will increase the level of spontaneous mutagenesis, and result in random mutations of oncogene and tumor suppressor loci in epithelial cells. Our long-term goal is to utilize this system to elucidate the role in breast cancer development of various endogenous conditions which may contribute to genetic instability, such as estrogen metabolism and oxidative stress, both of which form DNA adducts repaired by the BER pathway. This avenue of research is vital to understanding oncogenesis in the majority of sporadic human breast cancers, the etiology of which is not associated with familial genetic defects or gross exposures to environmental chemicals.

Body

I. Task 1: Isolation and characterization of polß overexpressing MCF-10A and -10AT cell lines.

During Year 1, we reported the establishment of the MCF-10A cell culture system and the selection of MCF-10A/pCDNApolß transfectants. We concluded last year's progress report by stating that we will perform experiments to optimize all of our conditions and that we will include the transformed cell line, MCF-10AT, in our analyses. We have accomplished all of these optimizations, the results of which are summarized in Table 1. In addition, we completed construction of the pIRES-polß expression vector (2001 Progress Report, Figure 2B). This vector proved to be superior over the pcDNA vector, and has been used in all experiments included in this Progress Report.

A. Optimization of cell transfection experiments

A significant impediment to our progress last year was the extremely slow growth rate of MCF-10A cells and the low efficiency of DNA transfection. We have since determined that the passage number (generation number) of MCF-10A cells has a profound influence on both of these parameters. As shown in Table 1, MCF-10A cells at a passage number greater than 100 displayed an enhanced efficiency of DNA transfection. In contrast, MCF-10AT cells, a *ras*-transformed derivative of MCF-10A, were highly efficient for DNA transfection, independent of passage number. A second variable which influenced MCF-10A transfection efficiency was the

Table 1. Optimization of MCF-10A and 10AT DNA transfection

Experimental Variable	Relative Transfection Efficiency	
	MCF-10A	MCF-10AT
1. Passage Number	P96: + P98: + P101: +++	P33: +++ P36: +++ P38: +++
2. Transfection Method		
Electroporation	+++	+++
Lipofection	++	++++
3. Growth Conditions		
Complete	+++	+++
Serum depleted	+	++
Growth factor and serum depleted	-	+
4. Vector		
pIRES	+++	++++
pIRES WTpolß	++	++

method for introducing DNA into the cells. Electroporation of the DNA was more efficient than lipofection using lipofectin reagent from several commercial sources. Interestingly, the opposite was observed for the transformed MCF-10AT cells, for which lipofection was more efficient than electroporation (Table 1). Other experimental procedures, such as serum starvation or growth factor deprivation, failed to positively impact transfection efficiency for either cell line (Table 1). Finally, we observed a difference in the recovery of puromycin-resistant, stable transfectants when using the parent pIRES vector *versus* the pIRES WTpolß vector. We attribute this difference to less efficient utilization of the IRES entry site located upstream of the puromycin-resistance message when the polß gene is present, resulting in reduced translation of the puromycin resistance protein.

B. Analyses of WTpolß expression in parental and stable pIRES transfected populations

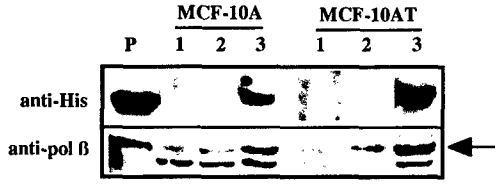

Figure 1. Polß expression in MCF-10A and 10AT cells transfected with pIRES vectors. Exogenous polß is detected by the anti-His antibody (top panel). Total cellular polß protein is detected by the anti-polß antibody (bottom panel). The polß form produced by the pIRES vector is indicated by the arrow. P, pure protein; lane 1, parental cells; lane 2, pIRES transfected cells; lane 3, pIRESWTpolß-transfected cells. Transfected cell populations were adherent cells, three passages post-selection.

During Year 1, we reported our initial, limited success at obtaining a polß-overexpressing cell population using the pIRES WTpolß vector and MCF-10A cells. The optimization experiments described above resulted in robust expression of exogenous polß in both MCF-10A and MCF-10AT cells (Figure 1, top panel, lane 3). Total polß protein is increased 1.8-fold in MCF-10A/ pIRES WTpolß cells and 2.8- fold in MCF-10AT/ pIRES WTpolß cells, relative to the corresponding pIRES controls (Figure 1, bottom panel, lane 2 *versus* lane 3).

C. Description of altered morphology with WTpolß overexpression

In our 2001 report, we described our experimental difficulties in obtaining a proliferative, polß-overexpressing MCF-10A cell population. During the second year, we continued to observe that both MCF-10A and MCF-10AT cells overexpressing WTpolß failed to proliferate as adherent cell populations three to four passages post-transfection (Figure 2). This failure was not observed in the pIRES vector transfected controls, indicating that the loss of the proliferative, adherent cell population is a result of polß expression. Recently, the non-adherent (floating) and adherent cell populations were independently followed for cell viability. As depicted in Figure 2, both the floating and adherent MCF-10A/WTpolß populations died after only a few doublings post-transfection. Similarly, the MCF-10AT/WTpolß adherent population lost viability at approximately P4. In contrast, a portion of the floating MCF-10AT/WTpolß population has retained viability through P3. In the original publication reporting the isolation of MCF-10A cells, Soule et al. described the isolation of both adherent (10A) and floating (10F) cell lines from the same MCF-10

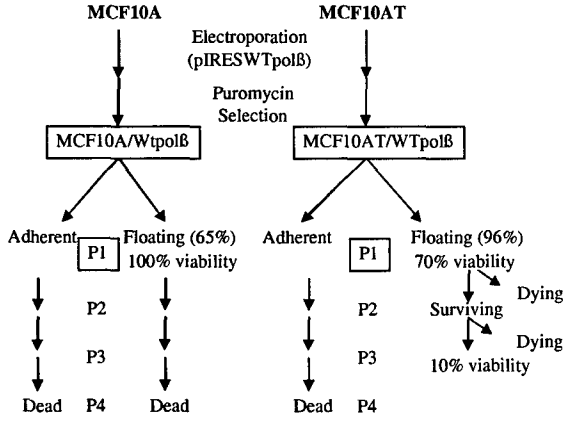

Figure 2. Schematic of phenotypic changes in MCF-10A and 10AT cell populations following polß overexpression.

cell progenitor. To our knowledge, however, no other reports have been published that describe the conversion of adherent (10A) to floating (10F) cells by gene overexpression. We will investigate this new phenomenon in greater detail during the next year.

II. Task 2: Quantitation of HSV-tk mutation rates

In our original proposal, we intended to quantitate the HSV-tk mutation rate in polß-overexpressing cell populations 30 and 60 passages post-transfection. Obviously, as illustrated in Figure 2, the observed loss of proliferative potential in MCF10A and MCF10AT cells expressing WTpolß preclude our ability to perform these mutational analyses. We have devised an alternative strategy to determine the HSV-tk mutation rate using a transient replication model system, which requires only 3-7 days of cell proliferation. Never-the-less, the observed changes in cell phenotype (adherent to floating) have cautioned us to postpone these mutational analyses until we are more certain of the characteristics of these two cell populations. In particular, we do not want to perform mutational experiments on a population with a high proportion of dead or apoptotic cells, as such data would be meaningless. Our current plan for this task is to derive separate floating and adherent polß expressing cell populations, followed by a transient mutational analysis.

III. Task 3: Determine *in vitro* transformation properties of polß overexpressing cell populations

In our original grant application, we had proposed to determine the extent of growth factor independence and the efficiency of anchorage-independent growth in polß expressing cell populations after ~30 and ~60 passages. Again, the lack of cell proliferation after 4 passages (Figure 2) has rendered us unable to accomplish this task to date. As an alternative strategy, we have undertaken the following studies to characterize the cell populations.

A. Molecular changes in transfected cell populations

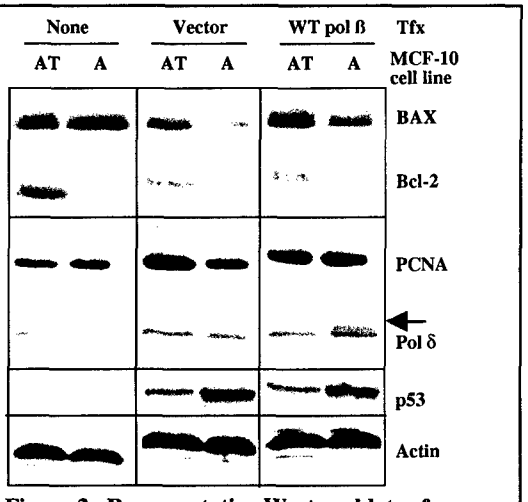

Figure 3. Representative Western blots of parental and MCF10A, 10AT transfected cell populations. Arrow indicates presence of a new molecular weight polδ species in MCF-10A cells.

We analyzed the degree to which puromycin selection and polß overexpression altered MCF-10A and MCF-10AT cells by Western analyses of a variety of molecular markers. A portion of these analyses are displayed in Figure 3 and summarized in Table 2. For all cases, we analyzed adherent cell populations three passages post-transfection.

MCF-10A cells are extremely sensitive to the toxic effects of puromycin, as described in our previous report. This cytotoxicity is reflected in the significant stabilization of p53 protein in the pIRES vector transfected MCF-10A cell population, relative to the untransfected parental cells (Figure 3). A much smaller effect of puromycin selection on p53 status in MCF-10AT cells was noted (two-fold), consistent with the transformed phenotype of this cell line. The overexpression of WTpolß, however, did not affect p53 protein levels in either cell line.

We also examined the relative expression of various DNA repair proteins, as polß is the rate-limiting enzyme in the base excision repair pathway. We examined proteins of the mismatch repair pathway (MSH2), which removes DNA polymerase errors, and the double strand break repair pathway (RAD50), which repairs DNA breaks causing chromosomal rearrangements. A notable increase in MSH2 protein level (4.2-fold) was observed in polß

overexpressing MCF-10A cells, relative to the vector control, but no such change was observed in MCF-10AT cells (Table 2). This result may be due to cellular compensation for an increased number of DNA mismatches caused by polß, and will be confirmed with further studies. No changes in RAD50 levels were observed for either cell line (Table 2).

Table 2. Phenotypic effect of WTpolß expression on MCF-10A and MCF-10AT cells: Western blot analyses

Pathway/Protein	Relative change of protein in polß-overexpressing cells [a]	
	MCF-10A	MCF-10AT
DNA Repair		
Mismatch repair / MSH2	4 x ↑	none
Double strand break repair / Rad50	none	none
Base excision repair /		
APE-1	none	none
PCNA	2 x ↑	2x ↓
pol delta	1.8 x ↑ *	none
Apoptosis		
p53	none	none
Bax	4 x ↑	2 x ↑
Bcl-2	not dectectable	none
Chromatin Structure		
RCC-1	3 x ↑	none

[a] Fold change relative to pIRES vector-transfected cells. Increases or decreases of less than 50% are indicted as "none".
* Indicates presence of new molecular weight protein species.

The levels of three other proteins in the base excision repair pathway were also examined: APE-1, PCNA, and DNA polymerase delta (pol δ). The most notable change in this pathway was the increased levels of PCNA and pol δ in MCF-10/WTpolß expressing cells, relative to vector controls, and the concomitant appearance of a new form of pol δ (Figure 3). The new molecular weight species may represent a phosphorylated form of the protein. These changes were not observed in MCF-10AT cell populations (Table 2 and Figure 3).

RCC-1 is a chromatin-associated protein implicated in nuclear formation, nuclear transport, transcription and DNA replication. The significance of the increased abundance of this protein in MCF10A/WTpolß cell populations (Table 2) is not known at this time.

B. Evidence for increased apoptosis in polß-overexpressing cells

A series of Western blots was performed in an attempt to determine the pathway(s) responsible for loss of proliferative potential in the adherent, polß-expressing cell lines. The relative abundance of the proteins Bcl-2 and Bax is known to affect the rate of apoptosis in cultured human cells. We can detect no anti-apoptotic Bcl-2 protein in any of the MCF10A cell lines (Figure 3). However, we have found a significant, four-fold increase in the levels of the pro-apoptotic Bax protein in MCF-10A cells expressing WTpolß, relative to vector controls (Table 2). Such a high level of Bax protein is consistent with the loss of proliferative potential of these cells after polß transfection (Figure 2). Unlike MCF-10A cells, MCF-10AT cells constitutively express detectable levels of Bcl-2 protein, as expected for *ras*- transformed cells (Figure 3). In pIRES and pIRESWTpolß transfected MCF-10AT cell populations, the levels of Bcl-2 are decreased, relative to the parental cells. Moreover, we have found an increase in the levels of Bax protein in the MCF-10AT/polß overexpressing cells (Table 2). The net effect is the alteration of the Bax:Bcl-2 ratio in polß overexpressing cells to favor the pro-apoptotic Bax (Figure 4). Functional studies are in progress to confirm that these molecular changes result in the induction of apoptosis in polß overexpressing cell populations.

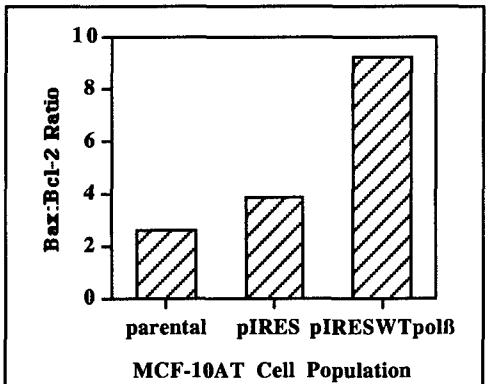

Figure 4. Evidence for increased apoptotic signalling in MCF-10AT/WTpolß expressing cells.

IV. Task 4: Tumorigenicity studies

A. Tumor incidence

Preliminary tumorigenicity studies were performed using parental cells and adherent, transfected cell populations three passages post transfection (Table 3). These are the same cell populations that were characterized by Western analyses (Table 2; Figures 3 and 4). As reported in the literature, MCF-10A cells did not form tumors when injected under the mammary fat pad of nude mice. The expression of WTpolß in these cells did not increase the incidence of tumor formation in this preliminary study.

Table 3. Effect of WTpolß overexpression on the tumorigenic potential of MCF-10A and MCF-10AT cells.

Cell line / vector	Number of nude mice	
	Injected[a]	Tumor-bearing[b]
MCF-10A	5	0
MCF-10A / pIRES	5	0
MCF-10A / pIRESWTpolß	8	0
MCF-10AT	5	4
MCF-10AT / pIRES	5	1
MCF-10AT / pIRESWTpolß	5	3

[a] Mammary fat pad injection of 1 x 10^6 cells three passages post-transfection

[b] Incidence after 175 days

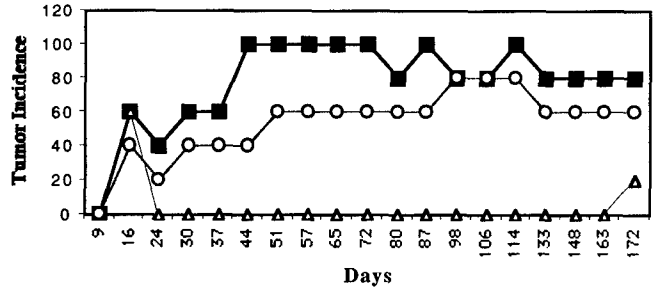

Figure 5. Tumor incidence in MCF-10AT cell populations. Squares, parental cells; Circles, pIRESWTpolß transfected, adherent cells; Triangles, pIRES transfected cells. All groups contained 5 animals.

The *ras*-transformed MCF10AT cell line is reported to be tumorigenic, and we observed a tumor incidence of 80% in this small study (Table 3). The tumors arose within 150 days after mammary fat pad injection (Figures 5 and 6). The incidence of tumors arising from MCF10AT/WTpolß cells was slightly lower than that of the parental cells while tumors arose with a similar latency period (Figure 5). A tumorigenicity study of MCF-10AT/WTpolß floating cell populations is in progress to determine whether the adherent→floating phenotypic change is associated with altered tumor potential.

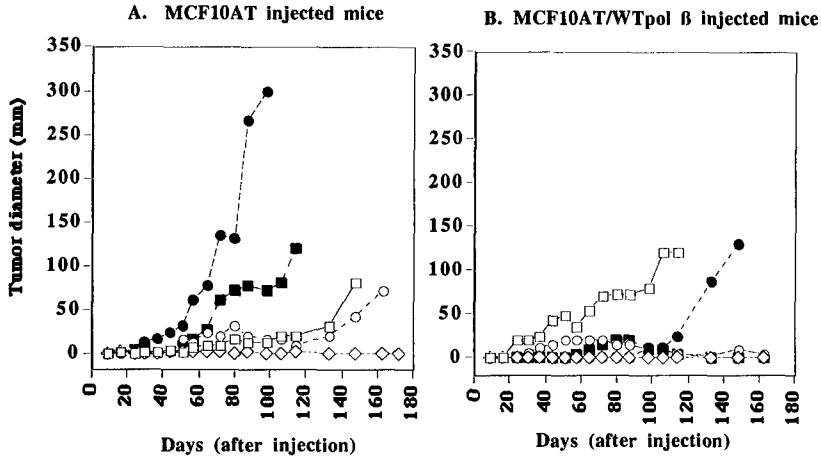

Figure 6. Tumor size per animal for MCF-10AT and MCF-10ATpIRESwtß groups. Each symbol represents an individual animal.

The tumors derived from parental MCF-10AT cells ranged in size from 100-300mm in diameter. In general, tumors derived from the polß overexpressing cells were smaller than those observed in the parental controls (Figure 6).

B. Tumor histology

The tumors arising in the MCF-10AT/pIRESWTpolß group were cyst-like and less solid than those arising from the MCF-10AT parental cells. Parrafin-embedded sections of all tumors have been made and all tumor tissue archived for future histological and molecular analyses.

V. Task 5: Metastasis studies

We hypothesized in our grant application that MCF-10AT-polß variant expressing cells will progress to acquire metastatic potential, a property which has rarely been observed in the MCF10A or 10AT parental cell lines. In our small study, we observed the presence of lung metastases in 2 of the 3 tumor-bearing mice derived from MCF-10AT/WTpolß expressing cells. In contrast, no lung metastases were observed among the four, tumor-bearing mice derived from the parental cells. Thus, these preliminary data are consistent with our hypothesis that increasing the mutation rate by increasing the levels of polß protein will accelerate neoplastic progression in human breast epithelial cells.

VI. Summary

Our research accomplishments associated with each task as outlined in the approved Statement of Work are summarized in Table 4.

Table 4. Summary of Research Tasks and Accomplishments (Year 2)

Approved Tasks (Year 1)	Individual Experiments	Progress to Date
Task 1: Isolate and characterize polß-overexpressing clones of MCF-10A and MCF-10AT cell lines (months 1-3)	**1A-1D.** MCF-10A: Reported in 2001 Annual Report; continuation in this report MCF-10AT: This report **1E.** Analyses of floating vs adherent cell populations	**Complete for WTpolß** **Table 1** **Figure 1** **Figure 2**
Task 2: Quantitate HSV-tk mutation rates in control and polß-overexpressing MCF-10A/AT cell lines (months 3-12)		**Postponed until Year 3** (section II)
Task 3: Quantitate *in vitro* transformation properties of control and polß-overexpressing MCF-10A/AT cell lines (months 9-18)	**3A.** Western blot characterization of transfected cell populations	**Complete for WTpolß** Figures 3 and 4 Table 2
Task 4: Quantitate mammary tumor incidence and latency in control and polß-overexpressing MCF-10A/AT cell lines (months 6-36)	**4A.** Monitor tumor development after mammary fat pad injection; quantitate tumor growth rate for each control and experimental cell line	**Partially complete** Table 3 Figures 5 and 6
Task 5: Observe metastatic potential of control and polß-overexpressing MCF-10A/AT cell lines (months 12-36)	**5A.** Determine the extent of remote metastases after mice become moribund, or after 1 year post-injection	**Partially complete**

Key Research Accomplishments

- Establishment of MCF-10AT cell culture system to complement the MCF-10A cell system.
- Isolation and characterization of WTpolß-overexpressing MCF-10A and MCF-10AT cell populations.
- Documented a phenotypic change from adherent to floating cell morphology in MCF-10AT cells after transfection with the pIRES polß expression vector.
- Characterized WTpolß-overexpressing cell populations with limited proliferative potential by Western analyses.
- Preliminary evidence found to support the hypothesis that polß overexpression alters the control of apoptosis in MCF-10A and MCF-10AT cells.
- Preliminary studies using nude mice demonstrating that polß overexpression does not increase tumor incidence or decrease tumor latency of MCF-10A or MCF-10AT cells.
- Preliminary studies using nude mice demonstrating that polß overexpression may increase the metastatic potential of MCF-10AT cells.

Reportable Outcomes

There were no reportable outcomes of this research during this reporting period.

Conclusions

We have observed that overexpression of DNA polymerase ß in a diploid, human breast epithelial cell line, MCF-10A, results in loss of proliferative potential, most probably due to the induction of p53 and the upregulation of pro-apoptotic proteins. The p53 response may, in part, be the result of antibiotic selection for the transfected cells. However, the increased levels of the Bax protein are specific for the increased levels of polß protein. The *ras*-transformed MCF-10AT cell line appears to be more tolerant of polß overexpression, possibly due to the increased levels of Bcl-2 protein in these cells. However, the MCF-10AT cells continue to display a progressive loss of proliferative potential resulting from polß overexpression. Unexpectedly, we observed an alteration in cellular phenotype from an adherent to a floating cell morphology in MCF-10AT cells after polß overexpression. To our knowledge, this is a novel observation and may be related to underlying genomic changes resulting from increased polß protein levels. The observation that MFC10AT cells overexpressing polß display a tumor incidence and latency similar to the parental cells is also intriguing. This observation implies that in contrast to what we observe *ex vivo* (e.g., in cell culture), a subpopulation of polß overexpressing cells retains proliferative potential *in vivo*. This observation has two possible explanations: either biologic selection occurs for a mutant, MCF-10AT/WTpolß cell variant within the mice, or the host environment of the mammary fat pad provides growth factors/hormones that allow the cells to proliferate. We favor the former explanation, and hypothesize that the genetic variation may be related to the adherent to floating morphology change. Also supporting the first explanation are the observations that the MCF-10AT/polß tumors are cyst-like rather than solid, and that animals bearing MCF-10AT/polß tumors progressed to form lung metastases. In-depth molecular examination of cells and tumors, as well as larger, more robust animal studies will be undertaken during the next year to explore these hypotheses. In addition, we will repeat these experiments using a mutator form of polß to ascertain whether altering cellular mutation rate alters the apoptotic response and/or tumorigenic potential of MCF-10A and MCF-10AT cell lines.

References

Soule, H.D., T.M. Maloney, S.R. Wolman, W.D. Peterson, R. Brenz, C.M. McGrth, J.Russo, R.J. Pauley, R.F. Jones, and S.C. Brooks (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10, Cancer Research 50: 6075-6086.